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APPLICATION NUMBER: *60/459,187*
FILING DATE: *March 31, 2003*

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04-01-03 60459187 03/31/03

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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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JC971 U.S. PTO 60/459187 03/31/03

INVENTOR(S)

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[X] Additional inventors are being named on the 1 separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

Topoisomerase Modulator Assays

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ENCLOSED APPLICATION PARTS (check all that apply)

[X] Specification Number of Pages 17
[X] Drawing(s) Number of Sheets 3
[ ] Application Data Sheet. See 37 CFR 1.76
[ ] CD(s), Number
[X] Other (specify) Return receipt postcard

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

[ ] Applicant claims small entity status. See 37 CFR 1.27.
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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Respectfully submitted,

SIGNATURE _____

TYPED or PRINTED NAME Robin S. Quartin

TELEPHONE 302-885-9129

Date 03/31/03

REGISTRATION NO. (if appropriate) 45,028

Docket Number: 100995-1 US

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TOTAL AMOUNT OF PAYMENT	($) 160.00

Complete if Known	
Application Number	not yet assigned
Filing Date	March 31, 2003
First Named Inventor	Maria Uria-Nickelsen
Examiner Name	not yet assigned
Group Art Unit	not yet assigned
Attorney Docket No.	100995-1 US

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FEE CALCULATION

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101	740	201	370	Utility filing fee	
106	330	206	165	Design filing fee	
107	510	207	255	Plant filing fee	
108	740	208	370	Reissue filing fee	
114	160	214	80	Provisional filing fee	160.00

SUBTOTAL (1) ($) 160.00

2. EXTRA CLAIM FEES

	Extra Claims		Fee from below	Fee Paid
Total Claims		-20** =		X =
Independent Claims		- 3** =		X =
Multiple Dependent				=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	84	202	42	Independent claims in excess of 3
104	280	204	140	Multiple dependent claim, if not paid
109	84	209	42	** Reissue independent claims over original patent
110	18	210	9	** Reissue claims in excess of 20 and over original patent

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FEE CALCULATION (continued)

3. ADDITIONAL FEES

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105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for *ex parte* reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	400	216	200	Extension for reply within second month	
117	920	217	460	Extension for reply within third month	
118	1,440	218	720	Extension for reply within fourth month	
128	1,960	228	980	Extension for reply within fifth month	
119	320	219	160	Notice of Appeal	
120	320	220	160	Filing a brief in support of an appeal	
121	280	221	140	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,280	241	640	Petition to revive - unintentional	
142	1,280	242	640	Utility issue fee (or reissue)	
143	460	243	230	Design issue fee	
144	620	244	310	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Processing fee under 37 CFR 1.17(q)	
126	180	126	180	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	740	246	370	Filing a submission after final rejection (37 CFR § 1.129(a))	
149	740	249	370	For each additional invention to be examined (37 CFR § 1.129(b))	
179	740	279	370	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	

Other fee (specify) _____

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SUBMITTED BY

Name (Print/Type)	Robin S. Quartin, Ph.D.	Registration No. (Attorney/Agent)	45,028	Complete (if applicable) Telephone	302-885-9129
Signature				Date	03/31/2003

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PROVISIONAL APPLICATION COVER SHEET
Additional Page

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Docket Number	100995-1 US

INVENTOR(S)/APPLICANT(S)		
Given Name (first and middle [if any])	Family or Surname	Residence (City and either State or Foreign Country)
Peter	Barth	Macclesfield, United Kingdom

Number 2 of 2

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DOCKET NO: 100995-1 US PROVISIONAL

TOPOISOMERASE MODULATOR ASSAYS

FIELD OF THE INVENTION

The present invention relates to screening assays for identifying compounds that modulate the activity of topoisomerase.

BACKGROUND

DNA topoisomerases all share the property of catalyzing interconversions between different topological forms of DNA. DNA topoisomerases have been isolated from plasmid, viral, prokaryotic, and eukaryotic sources. There are two classes of topoisomerase enzymes (termed type I and type II) that are distinguished by an operational difference; the type I enzymes catalyze DNA interconversions during which the linking number changes in steps of one, while the type II enzymes perform reactions during which the linking number changes in steps of two. Negatively supercoiled DNA is more easily unwound, allowing RNA polymerase to bind more readily to the DNA, hence promoting the transcription of certain genes (Reece & Maxwell, 1991, Crit. Rev. Biochem. Mol. Biol., 26:335-375).

DNA gyrase is a prokaryotic topoisomerase II composed of two separate subunits, encoded by the *gyrA* and *gyrB* genes. The GyrA protein functions in the breakage and reunion of DNA, while the GyrB protein has an ATPase activity. All topoisomerases are able to relax negatively supercoiled DNA, but only gyrase can also introduce negative supercoils into DNA.

Bagel *et al.* (1999, Antimicrobial Agents Chemother., 43:868-875) used the *gyrA* and *topA* promoters, in conjunction with the β-lactamase reporter gene, to measure the effect of mutants of gyrase and topoisomerase IV on the degree of DNA supercoiling in *E. coli* cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a coumermycin dose response curve using a plasmid containing the *dnaA* promoter operably-linked to the β-galactosidase reporter gene in *E. coli*.

Figure 2 shows a diagram of plasmid pBA704 (*recF* promoter operably-linked to *luxABCDE*).

Figure 3 shows the graphical results of various concentrations of various compounds on Lux expression in *S. aureus* using a plasmid containing the *recF* promoter operably-linked to the *lux ABCDE* operon (lux) reporter gene.

DETAILED DESCRIPTION

The present invention provides assays for identifying compounds that modulate the activity of topoisomerase. The assays are whole cell reporter assays using cells carrying DNA supercoiling-sensitive promoters that are transcriptionally fused or operatively linked to a reporter gene. Alteration in the expression of the reporter gene reflects the activity of the promotor that responds to changes in the topology of DNA due to the action of topoisomerase. Hence, compounds that modulate the activity of topoisomerase can be identified by virtue of a modulation in reporter gene expression.

Inhibition of topoisomerase activity will result in the alteration of DNA topology in cells that will result in an increase in expression of the reporter gene operatively linked to the topology-sensitive promoter. Enhancement of topoisomerase activity will result in the opposite effect on DNA topology and will result in a decrease in the expression of the reporter gene operatively linked to the topology-sensitive promoter. Thus, compounds that modulate topoisomerase activity are identified by an alteration in reporter gene expression.

Inhibition of DNA gyrase activity results in a reduction of DNA supercoiling, which results in an increase in expression of the reporter gene operatively linked to the topology-sensitive promoter. Enhancement of DNA gyrase activity results in an increase in DNA supercoiling, which results in a decrease in the expression of the reporter gene operatively linked to the topology-sensitive promoter. Thus, compounds that inhibit DNA gyrase activity are identified by an increase in reporter gene expression, and compounds that enhance DNA gyrase activity are identified by a decrease in reporter gene expression.

We have utilized the *dnaA* promoter operably-linked to the β-galactosidase reporter gene to develop a cell-based reporter assay in the Gram-negative bacterium *Escherichia coli*. We have also created a similar constructs containing the *recF* promoter operably-linked to either the *lux ABCDE* operon (lux) reporter gene for use in the Gram-positive bacterium *Staphylococcus aureus*. We have used these constructs to show that known gyrase inhibitors

can be identified by enhancement of the expression of reporter genes in both Gram-positive and Gram-negative systems.

The whole cell reporter assays of the present invention can be used to identify topoisomerase modulators using intact cells. More particularly, the assays of the present invention can be used to identify inhibitors of bacterial DNA gyrase for the development of antibacterial agents. The assays of the present invention can be carried out in both Gram-positive and Gram-negative bacterial systems, thereby allowing for the identification of broadspectrum inhibitors.

One aspect of the present invention is a method for identifying compounds that modulate topoisomerase activity. The method comprises (a) contacting cells that express a topoisomerase with a test compound, wherein the cells contain a promoter that is sensitive to changes in DNA topology and a reporter gene operably linked to the promoter, and (b) measuring the expression of reporter gene, where an alteration in reporter gene expression in the presence of the compound relative to the absence of the compound is indicative of a compound that modulates topoisomerase activity.

In some embodiments, the assays of the present invention are used to identify compounds that inhibit topoisomerase activity, wherein an increase in reporter gene expression in the presence of a test compound relative to the absence of the test compound is indicative of a test compound that inhibits topoisomerase activity.

In another aspect, the present invention provides a method for identifying compounds that modulate DNA gyrase activity, said method comprising (a) contacting cells expressing DNA gyrase with a test compound, wherein said cells contain a promoter sensitive to changes in DNA topology and a reporter gene operably linked to said promoter; and (b) measuring reporter gene expression, where an alteration in reporter gene expression in the presence of the compound relative to the absence of the compound is indicative of a compound that modulates DNA gyrase activity.

In some embodiments, the assays of the present invention are used to identify compounds that inhibit DNA gyrase activity, wherein an increase in reporter gene expression in the presence of a test compound relative to the absence of the test compound is indicative of a test compound that inhibits DNA gyrase activity.

As used herein, the terms "modulate" or "modulates" in reference to topoisomerase or DNA gyrase activity includes any measurable alteration, either an inhibition or enhancement, of topoisomerase or DNA gyrase activity. Assays of the present invention utilize reporter genes operably linked to promoters that are sensitive to changes in DNA topology as the

basis for detecting topoisomerase activity. Any measurable alteration in reporter gene expression can be correlated to a modulation of topoisomerase activity.

As used herein, the term "topoisomerase" refers to any topoisomerase from any source, including, but not limited to, topoisomerase I, topoisomerase II and DNA gyrase, topoisomerase III and topoisomerase IV. Topoisomerases have been identified in viruses, plasmids, prokaryotes, and eukaryotes. Any topoisomerase can be assayed using the methods of the present invention. For reviews on topoisomerase, see: Champoux, 2001, Annu. Rev. Biochem., 70:369-413; Wang, 2002, Nat. Rev. Mol. Cell. Biol., 3:430-440.

As used herein, the terms "DNA gyrase" and "gryrase" are used interchangeably to refer to DNA gyrase enzymes.

In some embodiments of the present invention, assays are used to identify compounds that modulate the activity of DNA gyrase. Any DNA gyrase can be tested in the assays of the present invention, including, but not limited to, DNA gyrase from members of the Enterobacteriaceae family such as *Escherichia coli, Salmonella spp*, and *Shigella spp*, and DNA gyrase from *Haemophilus influenzae*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa*, *Chlamydia spp*, *Legionella spp*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Enterococcus faecium*, and *Mycoplasma spp*.

In some embodiments of the present invention, the topoisomerase or DNA gyrase is assayed in the cells in which it is naturally expressed.

In some embodiments of the present invention, the topoisomerase or DNA gyrase is assayed in the cells in which it is not normally or naturally expressed, and the topoisomerase or DNA gyrase is recombinantly expressed in the assay cells. Thus, for example, mammalian topoisomerase may be assayed in bacterial cells, or one species of bacterial DNA gyrase may be assayed in another species of bacterial cell. Nucleic acids encoding topoisomerases have been cloned from many sources, including, but not limited to, bacteria, yeast, mammalian, and viral sources. Cloned mammalian topoisomerases include TopoI: human, mouse, rat, pig, Chinese hamster, dog, and chicken; TopoIIa: human, mouse, rat, pig, Chinese hamster, and bovine; TopoIIb: human, mouse, rat, pig, and Chinese hamster; TopoIIIa: human, mouse, and rat; and TopoIIIb: human and mouse.

Any cell type in which a topoisomerase or DNA gyrase is expressed or can be engineered to be expressed recombinantly, can be used in the assays of the present invention. Such cells include prokaryotic and eukaryotic cell types. Examples of such cells include, but are not limited to, bacterial, archeal, fungal (including *Aspergillus spp* and *Candida spp*), and mammalian (including human).

The terms "promoter sensitive to changes in DNA topology", "DNA topology-sensitive promoter" and "supercoiling-sensitive promoter" are used interchangeably to refer to polynucleotide sequences that are capable of promoting gene expression and that are responsive to changes in the topology of DNA. Any promoter that is sensitive to changes in DNA topology can be utilized in the methods of the present invention. At least one third of the promoters in *E. coli* are known to respond to changes in DNA topology in cells (Jovanovich & Lebowitz, 1987, J. Bacteriol., 169:4431-4435). Examples of bacterial promoters that can be used in the assays of the present invention include, but are not limited to, *gyrA, gyrB, proU, tppB, ompC, ompF, topA, dnaA* and *recF*.

The following promoter sequences and functional fragments thereof can be used in the methods of the present invention:

topA promoter:

5'CGGTCGATGGGTTGTGTCTCTTTGTTCATTATTTACTCCTTAAACAAGGACATTA
GTCTACGCCAGGCATGGCTTGCAGACAAATATACCACGCTGGTGGCAAGAGCGC
CTTACTGGCAACTTTGGATTTTGCATGCTAATAAAGTTGCGTATCGGATTTTATCA
GGTACAGTGTGACGCTTTCGTCAATCTGGCAATAGATTTGCTTGACATTCGACCA
AAATTCCGTCGTGCTATAGCGCCTGTAGGCCAAGACCTGTTAACTCAGTCACCTG
AATTTTCGTGAACAGAGTCACGACAAGGGGTTGATATCCGCAGAGAGCGAGTCC
ATATCGGTAACTCGTTGCCAGTGGAAGGTTTATCAACGTGCGACGCATTCCTGGA
AGAATCAAATTAGGTAAGGTGAAT 3' (SEQ ID NO:1)

gyrA promoter:

5'
TGGCACTTCTACTCCGTAATTGGCAAGACAAACGAGTATATCAGGCATTGGATGT
GAATAAAGCGTATAGGTTTACCTCAAACTGCGCGGCTGTGTTATAATTTGCGACC
TTTGAATCCGGGATACAGTAGAGGGATAGCGGTTAG 3' (SEQ ID NO:2)

S. aureus recF promoter:

5'
AAGGTGACGACTCGGTAACGCAATTAATTTTACCAATCAGAACTTACTAAAAATA
AATATAAATAAAGGATGACGTGATTAATTAAAACGTCATCCTTTATTTTTTGGCA
AAAATAATTCTAGATGCGTATGTAAAATAAATTTGACAGCATTTTAAACAGCAAA
TAAAAGACGCCAATTAAATTTATGACAAATGTATCCAAAATTTAATAAGTGTGCT
TATATGCCCTTTAAATTTAAAATTTTAATAGTCAATAACAAGTTGAATATTAAAG
TTAAACGCCGTTAAATAGCGTTAAAAAATTGAAAATGACAGTATTGCCAAAAAA
TAAGAATTAATTATTTATATGTAAACGGTTTCTACCTCTATTTTAAATGAAATTTG

TGACAAAAAAAGGTATAATATATTAATGACACACAAAGAAATGGAGTGATTATT
TTGGTTCAAGAAGTTGTAGTAGAAGGAGACATTAATTTAGGTCAATTTCTAAAAA
CAGAAGGGATTATTGAATCTGGTGGTCAAGCAAAATGGTTCTTGCAAGACGTTG
AAGTATTAATTAATGGAGTGCGTGAAACACGTCGCGGTAAAAAGTTAGAACATC
AAGATCGTATAGATATCCCAGAATTACCTGAAGAT 3' (SEQ ID NO:3)

E. coli dnaA promoter:

5'

GATCCTTATTAGATCGATTAAGCCAATTTTTGTCTATGGTCATTAAATTTTCCAAT
ATGCGGCGTAAATCGTGCCCGCCTCGCGGCAGGATCGTTTACACTTAGCGAGTTN
TGGAAAGTCCTGTGGATAAATCGGGAAAATCTGTGAGAAACAGAAGATC 3' (SEQ ID NO:4)

As used herein, the term "functional fragment thereof" in reference to a promoter sequence means any portion of an identified promoter sequence that retains the function of a promoter that is sensitive to DNA topology and functional in the assays of the present invention.

The promoter sensitive to changes in DNA topology and the reporter gene operably linked thereto can be provided in a variety of formats, including, but not limited to, on a plasmid, phage, cosmid, other DNA molecules, and integrated into the host cell chromosome.

In some embodiments of the invention, the promoter sensitive to changes in DNA topology and the reporter gene operably linked thereto are provided on a plasmid or autonomous, self replicating extrachromosomal piece of DNA that is maintained in the cells used in the assay. Any type of plasmid can be used with the assays of the present invention.

In some embodiments low-copy plasmids are used.

In some embodiments high-copy plasmids are used.

In some embodiments of the invention, the promoter sensitive to changes in DNA topology and the reporter gene operably linked thereto are provided on a chromosome in the cells used in the assay.

As used herein, the term "reporter gene" refers to any polynucleotide sequence that encodes a polypeptide product whose expression can be detected or measured. Reporter genes, their gene products, and methods for the detection or measurement of their expression are well known to those of skill in the art. Any of a wide variety of reporter genes whose expression can be detected or measured can be used with the assays of the present invention, including, but not limited to, *lacZ*, *lux*, *uidA*, *gfp* (green fluorescent protein), *phoA*, *kan*, and *cam*.

In some reporter systems the reporter gene product is measured or detected directly by virtue of, for example, chemiluminescent, fluorescent or light producing properties. In some reporter systems the reporter gene product is measured or detected indirectly via the detection or measurement of the activity of the reporter protein on a substrate.

The LacZ reporter protein can be detected by the addition of a reporter substrate: the chromophore signal results from the action of β-galactosidase on a colorless substrate (Miller, 1972, *Experiments in Molecular Genetics*, p 352-355, Cold Spring Harbor Press, N.Y.). The Lux reporter does not require addition of substrate, but relies on the intrinsic activity of luciferase for detection and can be measured directly when the *luxABCDE* operon is expressed (Francis et al., 2000, Infect. Immun., 68:3594-3600; Qazi et al., 2001, Infect. Immun., 69:7074-7082).

For the LacZ reporter system, such substrates as CPRG can be used, which when cleaved by LacZ, undergoes a change in its spectral properties that can be routinely measured. Other examples of reporter systems/substrates include, but are not limited to, the β-lactamase reporter system with a fluorescent/colorimetric β-lactam as a substrate and the phosphatase reporter system with a radio- or immuno-labeled phosphate substrate.

Reporter gene expression is monitored by the method appropriate to the particular reporter system used, including, but not limited to, visual inspection, fluorescence, radiography and others. For example, absorbance is measured for lacZ and luminescence is measured for Lux.

Assay conditions can be routinely optimized by those of skill in the art. Specific parameters for culture medium and growth conditions, reporter gene substrate, adjustments to maximize signal-to-noise ratio and linearity of signal will depend upon the cell type and reporter system used. Such adjustments of parameters are well within the skill of the art.

The invention is further illustrated by way of the following examples, which are intended to elaborate several embodiments of the invention. These examples are not intended to, nor are they to be construed to, limit the scope of the invention. It will be clear that the invention may be practiced otherwise than as particularly described herein. Numerous modifications and variations of the present invention are possible in view of the teachings herein and, therefore, are within the scope of the invention.

EXAMPLES

Example 1. Screening for supercoiling-sensitive promoters.

The T4 terminator (T4 t) and *lacZ* gene were inserted into a plasmid expression vector pTB244 to create the construct pSB2. In this construct, that transcription of the *lacZ* gene, without a promoter, is prevented by the upstream T4 terminator, such that insertion of a promoter in necessary for *lacZ* expression. A random *E. coli* DNA library was constructed by inserting *Escherichia coli* chromosomal *Sau*3A fragments into the *Bam*HI site of the screening vector pSB2, upstream of the promoter-less *lacZ* gene.

The library was transformed into the *lacZ*⁻ *E. coli* host strain MSD1011 (MM294 Δ*lac* = *E. coli* K12 *hsdR* Δ*lac*). Transformants, plated onto X-Gal medium, were found to be white or various shades of blue from light to dark. Blue transformants were screened for the presence of relaxation-stimulated promoters using microtiter plate ß-galactosidase assay.

The ß-galactosidase assays were carried out in 96-well, flat bottom microtiter plates. Blue (lac ⁺) ampicillin-resistant colonies of all sizes and shades of blue were purified, inoculated into 200 μl media (L-broth containing 50 μg/mL ampicillin) and incubated overnight at 37 °C. Cultures were then diluted 1:20 into fresh broth and incubated for 1.5 hours at 37 °C with shaking at 230 rpm to mid log phase. At this point half of each culture was transferred to a duplicate microtitre plate to which nalidixic acid had been added to give a final concentration of 200 μg/ml. Both plates were then incubated at 37 °C for 2 hours to allow expression of the reporter gene which was then assayed. Before doing the ß-galactosidase assay absorbances were read automatically on the Molecular Devices Microplate Reader at 595 nm in order to measure the differences in the cell titers between control and treated cultures. 25 μl of ß-galactosidase assay emulsion (prepared as follows: 5 mL ONPG (O-nitrophenyl-ß-D-galactopyranodside) at 4 mg/ml, 5 mL x1 Z-buffer, 0.3 mL 1 % SDS, 0.2 mL chloroform, 0.2 mL ether), was added to each well. The plates were read at 28 °C on a Molecular Devices Microplate Reader at 420 nm for 2 mins at 0.09 sec intervals.

The plasmid pRAS101, containing the *gyrA* promoter linked to a *lacZ* gene, was used as positive control (Carty & Menzel, 1989, Proc. Natl. Acad. Sci. USA, 86:8882-8886). pRAS101 has a reported stimulation of 3 – 4-fold when a culture containing it is treated with a gyrase inhibitor.

More than 1000 transformants were tested in duplicate. Clone 19.4 demonstrated a relaxation stimulated transcription ratio of 6.1. The majority of the tested transformants showed reduced expression after treatment with nalidixic acid. Less than 10 % demonstrated positive stimulation ratios, mostly ranging from about 1.5 – 2.0-fold, with pRAS101 (the positive control) giving an average of 3.1 fold.

Example 2. Screening for anti-gyrase compounds using a plate lawn technique.

The principle of the screen is based on the observation that if known gyrase inhibitors are spotted onto X-Gal medium containing bacteria which carry a DNA relaxation-stimulated *lacZ* gene on a plasmid, clear zones of inhibition surrounded by a blue ring are formed after overnight growth. The blue ring presumably forms at the drug concentration where optimum relaxation-stimulation of the reporter gene has occurred before the cells are killed.

The following three clones were used: a) clone pRAS101 containing the *gyrA* promoter linked to the ß-galactosidase gene; b) clone 19.4 (found by method described in section 3.3); and c) clone pSB2.his containing the *hisD* promoter linked to the ß-galactosidase gene.

Exemplary method

Cultures were grown overnight and diluted 1:1000 in (melted) L-agar cooled to 45 °C containing 50 μg/mL Ap and 60 μg/mL X-Gal. The medium was poured into 15 x 150 mm petri dishes immediately. After it had set, known gyrase inhibitors were applied automatically onto the solid agar surface using a Tomtec Quadra 96 machine as follows: 20 μg nalidixic acid (Nx), 100 μg novobiocin (Novo), 2 μg ciprofloxacin (Cipro), 10 μg coumermycin (Cou), 2 μg cinoxacin (Cinox), 2 μg pefloxacin (Pef), 2 μg fleroxacine (Fle), 2 μg flumequine (Flu) and 2 μg norfloxacine (Nor).

Negative controls were placed onto the surface as follows: 25 μg kanamycin (Km), 20 μg chloramphenicol (Cm), 15 μg tetracycline (Tc), 15 μg streptomycin (Sm), 50 μg rifampicin (Rif), 100 μg trimethoprim (Tri), 250 μg sulfathiazole (Su) and 25 μg polymixin B (Px).

Plates were incubated overnight at 37 °C.

In one experiment, four gyrase inhibitors and two control antibiotics were spotted onto a lawn of bacteria. Distinct blue rings developed after overnight growth due to increased expression of the DNA relaxation-stimulated reporter gene. Both control drugs (chloramphenicol and tetracycline) showed little to no stimulation. The three clones (pRAS101, 19.4 and pSB2.his) showed similar stimulation responses.

Example 3. *E. coli* supercoiling assay using the LacZ reporter.

Materials

Vector construct

The *dnaA* promoter (SEQ ID NO:4, above) was cloned into the *Bam*HI site of the screening vector pSB2 (described in Example 1 above), upstream of the promoter-less *lacZ* gene.

Part I: Growth of *E. coli* cells

A. 250 mL glass flasks
B. Aerobic incubator (37 °C)
C. P-2, P-10, P-200, P-1000 pipettes
D. Sterile pipette tips
E. LB broth
F. Frozen stock of cells
G. Sterile loops
H. Shakers

Part II: Assay

A. P-2, P-10, P-200, P-1000 pipettes
B. DMSO
C. Sterile pipette tips
D. LB broth
E. Compounds at 10X the concentration in wells contained in 10% DMSO
F. Microcide inhibitor (phe-Argβ-naphthylamide, Sigma P-4157)
G. 96-well plates
H. CPRG (2mg/mL)
I. Z buffer (see below)
J. Plate shaker (30°C)
K. Plate spectrophotometer

Z buffer preparation:

Z buffer, adjusted to pH 7, contains in 1 L:

A. 16.1 g $Na_2HPO_47H_20$
B. 5.5 g $NaH_2PO_44H_20$
C. 0.75 g KCl
D. 0.246 g $MgSO_47H_20$
E. 2.7 mL β-mercaptoethanol

Procedure:

Part I: Growth of *E. coli* cells

Day 1

1. Frozen culture was inoculated onto LB.
2. The culture was incubated at 37 °C overnight.

Day 2

1. 25 mL of broth was inoculated in a 250 mL flask.
2. The flask was incubated overnight at 37 °C, 150 RPM.

Day 3

1. The overnight culture was diluted 1:100 in LB.
2. The culture was incubated at 37 °C and 150 RPM until $OD_{600} = 0.1$.
3. The culture was diluted 1:10 in LB.
4. 45 µL of the 1:10 LB culture (step 3) was pipetted into each well of a 96 well-plate containing 5 µL of two-fold dilutions of the test compound in 10% DMSO. The last well of each row contained only 5 µL of 10% DMSO.
5. The plate was incubated at 30°C and 200 RPM for 2 hours.
6. 50 µL of CPRG (2mg/ml) and 100 µL of Z buffer was added into all the wells.
7. The plate was incubated at 30°C and 100 RPM overnight.
8. The OD_{570} was measured and the OD_{570} accounting for the increase in cell mass was subtracted (this value was obtained from a different row in the plate containing all the same ingredients as the other rows except that water was used instead of CPRG) to arrive at "corrected OD_{570}".
9. The "corrected OD_{570}" numbers were used to plot the IC_{50} curves.

The results with coumermycin are presented in Figure 1.

Example 4. *S. aureus* supercoiling assay using the Lux reporter.

1. The *S. aureus* strain (RN4220 or ARC516), containing plasmid pBA704 (recF promoter:luxABCDE) (see Figure 2), was incubated overnight at 37 °C, 230 rpm in tryptic soy broth (TSB) with 7 µg/mL chloramphenicol.

2. The overnight culture was diluted in TSB + 7 µg/mL chloramphenicol to OD600 0.02, and grown at 37 °C, 230 rpm until OD600 reached 0.3.

3. A 96-well plate (Costar solid white flat bottom, cat# 3600) was set up with TSB and test compounds as follows:

- A 200 µL aliquot of TSB, containing a test compound (final concentration ~32X MIC), was placed into the first well.
- 100 µL of TSB was aliquoted into wells 2 through 12.
- Two-fold serial dilutions of the test compound were transferred from well 1 across the plate to well 12.
- 100 µL was removed from well 12.
- 50 µL of *S. aureus* strain (OD600 0.3) was added to each well containing compound.
- One additional well was prepared without compound by mixing 100 µL TSB with 50 µL of *S. aureus* strain (OD600 0.3).

4. A 96-well plate (Costar black, flat bottom, cat# 3711) was set up with TSB and test compounds as follows:

- A 200 µL aliquot of TSB, containing a test compound (final concentration ~32X MIC), was placed into the first well.
- 100 µL of TSB was aliquoted into wells 2 through 12.
- Two-fold serial dilutions of the test compound were transferred from well 1 across the plate to well 12.
- 100 µL was removed from well 12.
- 50 µL of *S. aureus* strain (OD600 0.3) was added to each well containing compound.
- One additional well was prepared without compound by mixing 100 µL TSB with 50 µL of *S. aureus* strain (OD600 0.3).

5. The plates were incubated at 37 °C, 230 rpm for up to 3 hours.

6. The luminescence of the cells was measured in the white Costar plate on a Tecan Ultra Evolution using a 200 msecond integration time.

7. The OD_{492} of cells in the black Costar plate was measured on a Tecan Ultra Evolution.

8. The relative light units per OD of cells for each well was calculated and the results were plotted as RLU/OD vs compound concentration.

The results are presented in Figure 3.

The foregoing examples are meant to illustrate the invention and are not to be construed to limit the invention in any way. Those skilled in the art will recognize modifications that are within the spirit and scope of the invention.

We claim:

1. A method for identifying compounds that modulate topoisomerase activity, said method comprising:

a) providing cells expressing topoisomerase and containing a promoter sensitive to changes in DNA topology having a reporter gene operably linked thereto;

b) measuring the expression of said reporter gene;

c) contacting said cells with a test compound;

d) measuring the expression of said reporter gene in the presence of said compound;

e) comparing the expression of said reporter gene in the presence of said compound with the expression in the absence of said compound, and

f) identifying a compound that modulates topoisomerase activity as one that yields an alteration in reporter gene expression in the presence of the compound relative to expression in the absence of the compound.

2. The method of claim 1, wherein the topoisomerase is a type II topoisomerase.

3. The method of claim 1, wherein the topoisomerase is a DNA gyrase.

4. The method of claim 1, wherein the topoisomerase is a recombinant topoisomerase.

5. The method of claim 1, wherein the topoisomerase is a prokaryotic, eukaryotic, or viral topoisomerase.

6. The method of claim 1, wherein the promoter is selected from:
gyrA, gyrB, proU, tppB, ompC, ompF, topA, dnaA, recF, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and any functional fragment thereof.

7. The method of claim 1, wherein the cells are bacterial cells.

8. The method of claim 7, wherein the cells are Gram-positive bacterial cells.

9. The method of claim 7, wherein the cells are Gram-negative bacterial cells.

10. The method of claim 7, wherein the cells are selected from *Haemophilus influenzae*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa*, *Chlamydia spp*, *Legionella spp*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Enterococcus faecium*, and *Mycoplasma spp*.

11. The method of claim 1, wherein the cells are eukaryotic cells selected from mammalian or fungal cells.

12. The method of claim 11, wherein the cells are human cells, *Aspergillus spp*, or *Candida spp* cells.

13. The method of claim 1, wherein the promoter and reporter gene are provided on a plasmid.

14. The method of claim 13, wherein the plasmid is a low copy plasmid.

15. The method of claim 1, wherein the promoter and reporter gene are provided on a chromosome.

16. The method of claim 1, wherein the reporter gene is selected from *lacZ*, *lux*, *uidA*, *gfp*, *phoA*, *kan*, and *cam*.

17. The method of claim 16, wherein the reporter gene is *lacZ* or *lux*.

18. A method for identifying compounds that modulate DNA gyrase activity, said method comprising:

a) providing cells expressing DNA gyrase and containing a promoter sensitive to changes in DNA topology having a reporter gene operably linked thereto;

b) measuring the expression of said reporter gene;

c) contacting said cells with a test compound;

d) measuring the expression of said reporter gene in the presence of said compound;

e) comparing the expression of said reporter gene in the presence of said compound with the expression in the absence of said compound, and

f) identifying a compound that modulates DNA gyrase activity as one that yields an alteration in reporter gene expression in the presence of the compound relative to expression in the absence of the compound.

19. The method of claim 18, wherein the DNA gyrase is a recombinant DNA gyrase.

20. The method of claim 18, wherein the DNA gyrase is selected from: *Haemophilus influenzae*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa*, *Chlamydia spp*, *Legionella spp*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Enterococcus faecium*, and *Mycoplasma spp*.

21. The method of claim 20 wherein the DNA gyrase is *E. coli* or *S. aureus* DNA gyrase.

ABSTRACT

The present invention provides methods for screening for compounds that modulate the activity of topoisomerase.

FIGURE 1

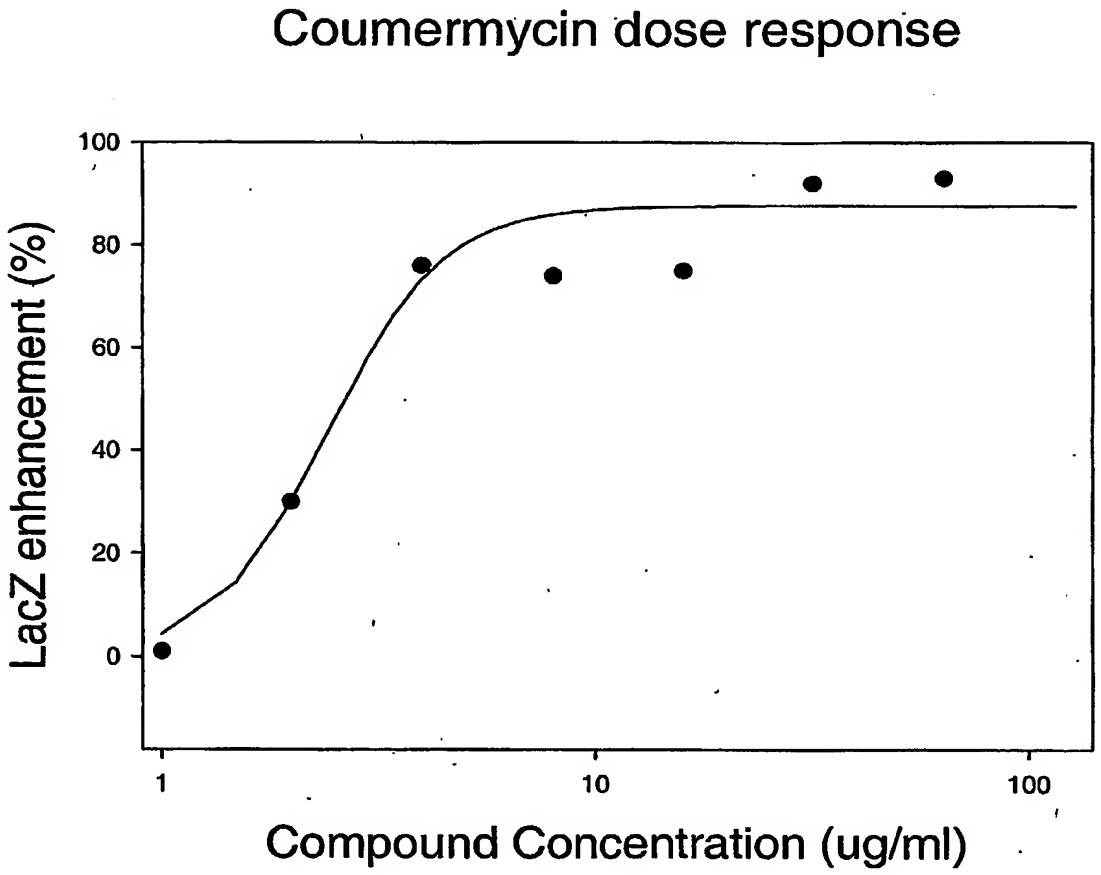

Coumermycin dose response
LacZ enhancement (%)
100
80
60
40
20
0
1
10
100
Compound Concentration (ug/ml)

FIGURE 2

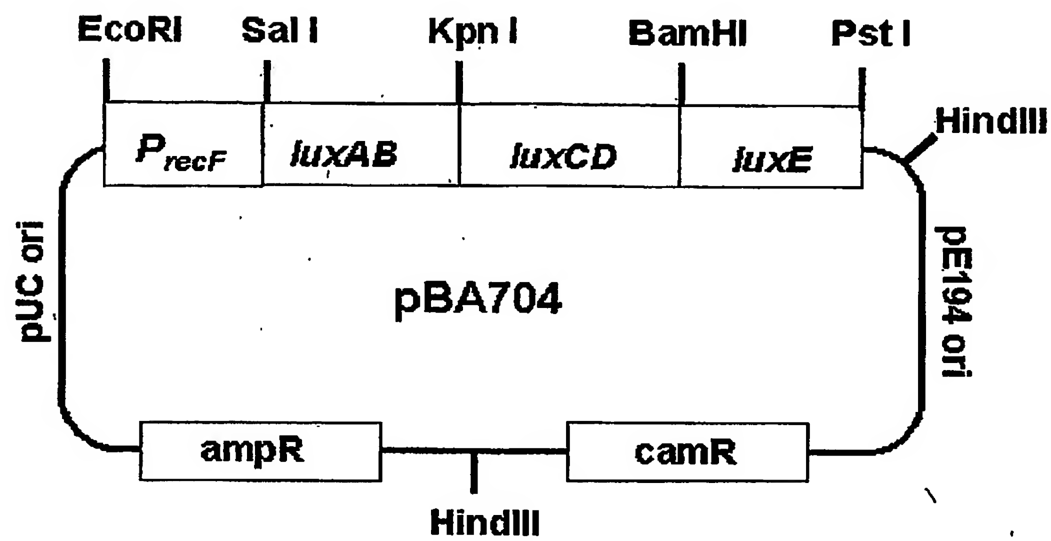
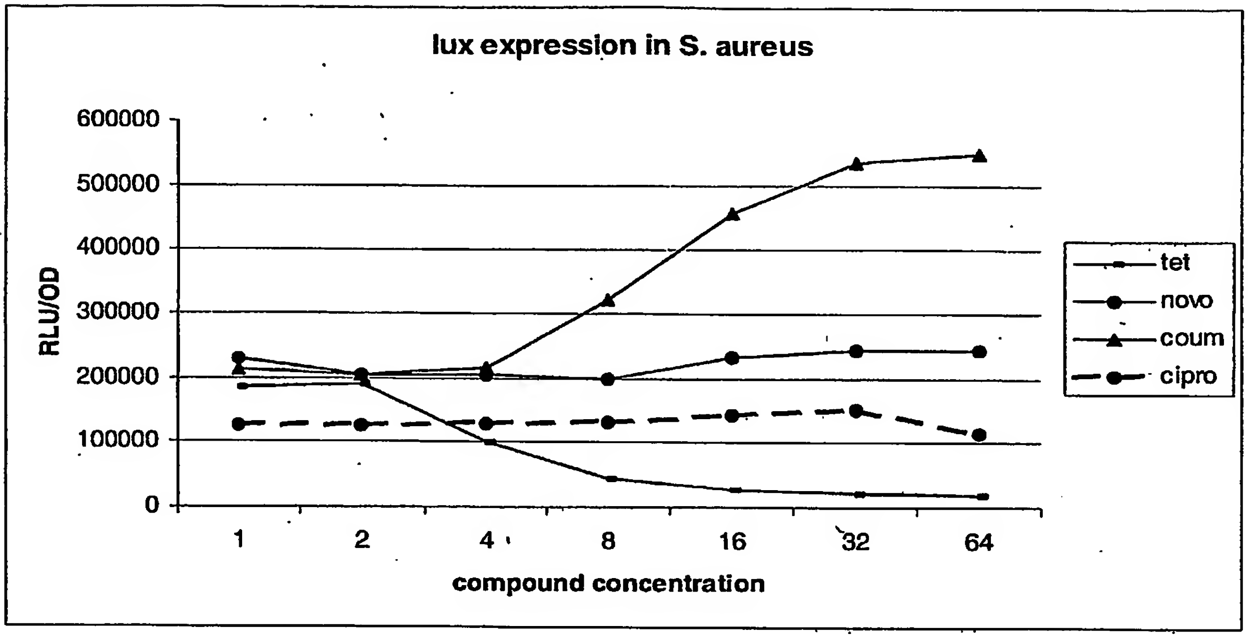

EcoRI
Sal I
Kpn I
BamHI
Pst I
HindIII
PrecF
luxAB
luxCD
luxE
pUC ori
pBA704
pE194 ori
ampR
camR
HindIII

FIGURE 3


lux expression in S. aureus
RLU/OD
600000
500000
400000
300000
200000
100000
0
1
2
4
8
16
32
64
compound concentration
tet
novo
coum
cipro